Chapter 3

Sex differences in yolk hormones depend on maternal social status in Leghorn chickens (Gallus gallus domesticus)

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Abstract

Maternal hormones are known to be present in avian eggs and can have beneficial effects on chick development. Recently, differences in avian yolk steroid concentrations between the sexes have been demonstrated, and in this context steroids have been hypothesized to be part of the avian sex-determining mechanism. In our study we clearly show that it is very unlikely that androgen concentrations alone are the decisive part of the sex-determining mechanism. We found that sex-specific differences in the yolk hormones strongly depend on the social rank of the mother. First, dominant females, but not subdominant females, allocated significantly more testosterone to male eggs than to female eggs. Second, subordinate females increased the testosterone concentrations of female eggs.

This pattern of yolk hormone deposition can be functionally explained. In polygynous species as the chicken, reproductive success is more variable in males than in females. Parental investment in sons or daughters is therefore expected to occur in direct relation to parental rearing capacities. Social status of a hen was in our study indeed negatively correlated with her maternal capacities (body mass, egg mass). Differential androgen deposition might thus provide a mechanism for adaptive maternal investment depending on both sex of the egg and social status of the mother.

Introduction

Avian eggs contain hormones of maternal origin. These hormones seem to reflect the hormonal state of the female during egg production (Schwabl 1996a, 1997a) and have been shown to influence the development and phenotype of the offspring (Adkins-Regan *et al.* 1995, Schwabl 1993, 1996b, Lipar & Ketterson 2000). In particular maternal androgens are known to increase competitiveness in the nestling as well as in the juvenile stage through higher aggressiveness and enhanced growth rate (Schwabl 1993, 1996 b, Eising *et al.* 2001).

Systematic variation of maternal hormones both within clutches (e.g. Schwabl *et al.* 1997b, Gil *et al.* 1999, Lipar *et al.* 1999, Eising *et al.* 2001, French *et al.* 2001, Royle *et al.* 2001) and between clutches (e.g. Schwabl 1996, 1997a, Gil 1999, Groothuis & Schwabl 2002, Wittingham & Schwabl 2002) has been reported. The first has mainly been discussed as a possibility for the mother to compensate for detrimental effects of hatching asynchrony (Schwabl 1996a, Lipar & Ketterson 2000, Eising *et al.* 2001, but see also Sockman & Schwabl 2000). The second level of variation suggested that social factors have a strong impact on androgen levels of a clutch [social environment: Schwabl (1996, 1997a), Reed & Vleck (2001), Groothuis & Schwabl (2002), Wittingham & Schwabl (2002); male attractiveness: Gil *et al.* (1999)].

In addition, Petrie *et al.* (2001) recently showed that for the peafowl (*Pavo christatus*) yolk steroids were also allocated differentially in relation to the sex of the embryo. Since the yolk is provided with hormones before the sex is determined (Sturkie 1986), Petrie *et al.* (2001) suggested that the steroids in the yolk itself may influence sex-chromosome segregation at the first meiotic division. Thus, sex specific differences in yolk hormone concentrations might be a consequence of these processes.

However, we consider four possible confounding factors in Petrie et al.'s study. First, hormone levels in the peafowl were determined after 10 days of incubation and might therefore not represent maternal hormone allocation. This is supported by the results of Schwabl (1993) who found no sex specific androgen deposition in freshly laid eggs of the canary. Especially in size-dimorphic species (like the peafowl and other *Galliformes* like our study species) male and female embryos may consume maternal steroids in a different rate. Second, endogenous production of sex steroids of male and female embryos differs (Woods *et al.* 1975) and subsequently may be passed through into the yolk (Jennings *et al.* 2000). With an increase of incubation time both might lead to a (secondary) sex difference in yolk hormones. Third, housing four peahens with a male in a cage, like in the study of Petrie *et al.* (2001), most likely leads to an establishment of a social hierarchy (e.g. Banks 1956, Guhl 1962). Since it has been shown that the female endocrine state varies with her social position (Allee *et al.* 1939, Frank *et al.* 1985, Batty *et al.* 1986), we expect that this effect might be found back in the eggs via the yolk hormones. Yolk hormone contents of different hens within one

group might therefore vary systematically. Because number and weight of eggs are not evenly distributed over the different ranks (Leonard & Weatherhead 1996), a random selection of single male/female eggs of one cage without taking the female into account might lead to a biased sample.
In addition social factors might cause sex-specific within clutch variation in yolk androgens. In polygynous mating systems as in *Galliformes*, male mating success is more variable than female mating success (e.g. Guhl & Warren 1946, Graves *et al.* 1985, see also Clutton-Brock *et al.* 1984). Hence, optimal parental investment should be different for the sexes, depending on parental rearing capacities (Trivers & Willard 1973). Since social status has been shown to positively affect maternal rearing capacities (Collias *et al.* 1994, Leonard & Weatherhead 1996), dominant females should invest more in male than female offspring whereas subordinate females should invest more in daughters. This might result in a shifted sex ratio or lead to a differential resource allocation e.g. of yolk androgens that provide a mechanism to influence offspring phenotype.
To evaluate these potential confounding factors that might have influenced Petrie et al.'s study we conducted an experiment were these factors were taken into account. We measured yolk androgen concentrations in eggs of White Leghorn chickens (*Gallus gallus domesticus*) in relation to the sex of the embryo after only three days of incubation. Thereby we minimized the possibility of secondary sex-specific processes affecting yolk hormone concentrations. This allows us to interpret any sex specific differences in yolk hormones as really reflecting maternal allocation. In addition we also investigated the relationship between social rank of the mother and yolk androgen deposition.

Material and Methods

(a) Animals

25 individually color ringed White Leghorn chickens were housed in five groups of four females and one male for about two months before the experiment started. Thus a stable social hierarchy was established at the beginning of our experiment. Each pen was 5 x 10 m with outside and inside areas [natural daylight 12:12 h (march)]. The outside parts of pens were separated only by a chicken wire partition. Thus, acoustic and optical but no direct contact between groups was possible. Food and water was provided *ad libitum*. Every pen contained a single nest box where all hens laid their eggs. Only a single female could enter this box at one time to lay her egg. At the start of the experiment all females were weighed.

(b) Behavioral observations

The dominance hierarchy within each group was investigated by daily 20-min observations for a period of three weeks. All observations were conducted at the same time of the day (13.00-15.00 h), mostly combined with feeding of favored

food (e.g. mealworms). The order in which the cages were observed was randomized each day. During the feeding all agonistic interactions between the females were recorded. The dominance hierarchy was based on the proportion of winning or loosing interactions with other females. A female lost an interaction if she fled when another hen pecked, chased or threatened her. Subsequently the birds were ranked (highest rank: 1, dominant; lowest rank: 4, subordinate) according to which individuals they dominated. A hen was considered to be dominant to another if it won more interactions than it lost with that hen. In addition the percentage of feedings in which a hen obtained a share of the offended food was scored.

(c) Egg collection

During the light period of the 3 weeks of the experiment it was registrated, which female entered the nest box. After a hen had left again, we checked instantly if an egg was laid. The egg was then removed and individually marked with a non-toxic marker. At the end of the day all eggs were measured and weighed and subsequently placed in an incubator at 37.5 °C with 60 % humidity. After incubation for 72 h all eggs were weighed again and stored at –20 °C. In total 182 eggs were collected. Laying order can not be a confounding factor in this study since the eggs of each hen were collected randomly with respect to day within the 3 week period. Laying order is also unlikely to be important in our birds since the hens lay eggs almost every day of the year, and not in separate clutches.

(d) Molecular analyses

For molecular analysis the collected eggs were defrosted and subsequently yolk and embryo separated. Both were separately prepared for 1. Molecular sexing (embryo) and 2. Androgen assays (yolk). The embryos were placed in eppendorff® tubes containing 100% ethanol and refrozen at –20 °C. The yolks were homogenized with 1 ml water per gram of yolk and stored again at –20 °C till the analysis.

Because we were interested in sex differences, only fertilized eggs were used for further measurements (n=120). Unfortunately in one of the groups the cock was infertile, therefore this cage was excluded from the analysis of yolk hormones and sex ratios. Data of all 5 cages were used for all other analyses.

I. Molecular sexing

About 1 µg tissue of each embryo was used for Chelex® resin-based DNA extraction (Walsh *et al.* 1991). Subsequently 2 µl of the obtained DNA solution was used for the polymerase chain reaction (PCR) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (for details see Griffiths *et al.* 1998). The amplified products were separated in 2.5 % agarose gels containing

0.005 % ethidiumbromide and subsequently visualized under UV light. Based on the presence of the PCR-products embryos were assigned to be male (CHD-Z gene product only) or female (CHD-Z gene as well as CHD-W gene products). The method has been developed and validated for domestic chickens (Griffiths *et al.* 1998) and we checked it on the basis of all our adult birds, finding a 100% correct outcome.

II. Androgen assays

When available, at least 3 male eggs and 3 female eggs per hen were selected for hormone analysis. These eggs were randomly chosen throughout the laying period. The selected samples were defrozen and about 150 mg of the yolk/water emulsion was used for the subsequent analysis. All extraction and radioimmunoassay were done following a slightly modified standard procedure according to Schwabl (1993). In short, samples were extracted twice with 4 ml petroleum ether/diethylether (30/70%), followed by precipitation with 90% ethanol to remove neutral lipids. Subsequently the hormones were separated on diatomaceous earth chromatography columns. Androstenedione and testosterone concentrations were measured in double competitive binding radioimmunoassays (RIA) with tritiated hormone (NEN, The Netherlands) and hormone specific antibodies (Endocrine Science, USA). The average recovery was 66% for androstenedione and 50% for testosterone. The inter-assay coefficients of variation were 17% for androstenedione and 11% for testosterone, intra-assay variation was 15% for androstenedione and 14% for testosterone.

(e) Statistical analyses

Testosterone but not androstenedione concentrations were normally distributed. For androstenedione therefore log-transformed values were used in our analysis. Yolk androgen levels, egg weight, yolk weight and embryo weight were analyzed using hierarchical linear modeling in the MLwiN program 1.1 (Rasbash *et al.* 2000). This method allows analyses of variances and co-variances considering the nested relationship of different chickens in a cage and repeated measures of the same hen. Significance was based on a two-tailed t-test. The following variables were tested in a backward elimination procedure: social rank, sex (of the embryo), female body weight, egg weight, yolk weight, number of eggs laid and all possible interactions. Only variables that contributed significantly ($\alpha \leq 0.05$) to the model were maintained. P-values of these variables are presented in the text. Posthoc analyses were performed for subsamples using the same test.

Statistical analyses of female body weight, number of eggs laid by a hen and the sex ratio in relation to the social status of the hen were performed using Linear Regression and Multiple Logistic Regression (in case of sex ratios) (Statistix® 7, Analytical software 2000).

Results

(a) Social Hierarchy

Within a cage all females could be ranked according to the number of fights won. This resulted in a linear hierarchy, were the highest rank (rank=1) dominated all other females and the lowest rank (rank=4) was subordinate to all other females (table 1). In addition to the outcome of direct interactions, which are the main data for the determination of social dominance, all results were confirmed by the frequency that a hen obtained food during a feeding trial (table 1).

cage	rank	1	2	3	4	frequency	female weight
6	1		27/31	6/6	6/6	100	1915
	2			7/7	6/6	75	1815
	3				1/1	25	1886
	4					13	1698
7	1		10/13	11/17	8/11	100	2084
	2			21/27	14/23	100	1639
	3				26/36	100	1642
	4					0	1512
8	1		6/6	2/3	3/3	100	1775
	2			9/9	32/48	92	1706
	3				11/16	25	1560
	4					8	1510
9	1		35/35	66/67	24/30	100	1570
	2			49/55	12/13	75	1543
	3				6/6	75	1626
	4					0	1220
10	1		18/21	21/33	14/18	80	1968
	2			12/21	4/5	60	1956
	3				2/2	0	1988
	4					0	1513

Table 1: Social dominance hierarchy for the five cages based on direct interactions between the hens (Number of won fights/Number of fights) and participation during the feeding (% of feedings that a hen got at least one food item).

Dominant females were heavier than subdominant females (N=20 females, r^2=0.33, p<0.01). The egg weight of a specific hen was positively correlated with her body weight (p<0.01) and thus negatively with social status (p<0.05). Since yolk weight and egg weight were positively correlated (r^2=0.15, p<0.001), the same pattern exists for yolk weight (body weight: p<0.001; social status: p=0.01). Embryo weight was not correlated to any variable included in the model. Furthermore, neither social rank nor body weight had an effect on the number of eggs that were laid (body weight: r^2=0.11, p= 0.15; social status: r^2=0.02, p=0.58) and both were not correlated with the sex ratio produced by a hen (body weight: $F_{1,13}$=0.29, p=0.58; social status: $F_{1,13}$=0.58, p=0.54) (for details see table 2a).

status	total no. of females	mean body weight	mean egg weight	mean no. of eggs	total no. of eggs	total no. of fertilized eggs	total no. of females	sex ratio
1	5	1862.4+/-88.3	66.78±0.69	9.6±2.3	48	34	4	0.64
2	5	1731.8+/-71.4	67.92±0.46	10.4±1.5	52	40	4	0.48
3	5	1740.5+/-82.9	63.69±1.44	8.0±3.0	39	22	3	0.43
4	5	1502.6+/-78.4	63.49±0.57	8.6±2.2	43	24	3	0.44
sum	**20**				**182**	**120**	**14**	

Table 2a: Female body weight and characteristics of her eggs in relation her social status (mean +/-std. error).

		Testosterone				Androstenedione			
		males		females		males		females	
status	females	no eggs	concentration	no eggs	concentration	no eggs	concentration	no eggs	concentration
1	4	14	1.71±0.12	11	1.08±0.14	14	37.89±5.18	11	33.70±5.10
2	4	14	1.23±0.15	14	1.26±0.16	14	38.32±5.22	14	37.45±7.07
3	3	7	1.53±0.16	9	1.79±0.17	7	29.86±8.76	9	32.05±4.52
4	3	9	1.86±0.28	12	2.34±0.28	8	41.20±12.05	12	44.61±10.60
sum	**14**	**44**		**46**		**43**		**46**	

Table 2b: Androgen concentrations for male and female eggs in relation to the social status of the mother (mean+/-std. error).

(b) Yolk androgens

In total, 90 eggs of 14 different females were analyzed for yolk hormone concentrations (for details see table 2b). If we used sex of the embryo as the only factor in the model, we did not find a significant difference in either yolk testosterone or androstenedione concentrations between the sexes (testosterone: p=0.95, androstenedione: p=0.98) (figure 1).

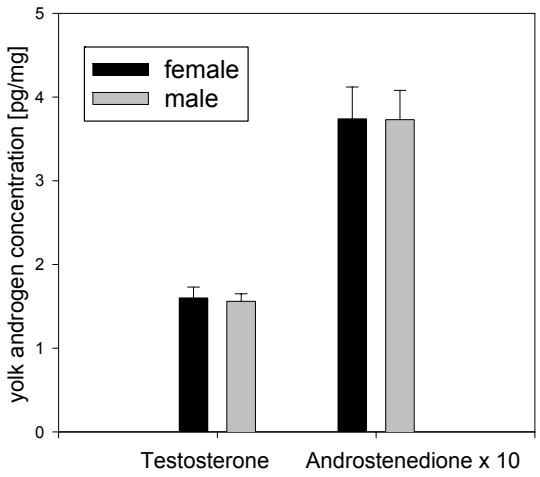

Figure 1: Yolk androgen concentrations (in pg per mg of fresh yolk) in male (N=44) and female (N=46) eggs of White Leghorn chickens (mean +/-std. error).

In the final model a relationship between yolk testosterone levels and social status of the hen (p=0.009), sex of the embryo (p=0.002) and the interaction of these two variables (sex*rank: p=0.001) was retained. We found that with decreasing social position the testosterone concentrations in the yolk of

daughters increased (post hoc test, $p<0.001$), but did not change in sons (post hoc test, $p=0.98$). Moreover, in eggs of dominant females (rank 1), sons had significantly higher testosterone concentrations compared to daughters (post hoc test, $p=0.001$) (figure 2). In eggs of subdominant hens female eggs had somewhat higher testosterone concentrations compared to male eggs. However this sex effect did not reach statistical significance (Rank 2-4, $p\geq0.13$ in all cases).

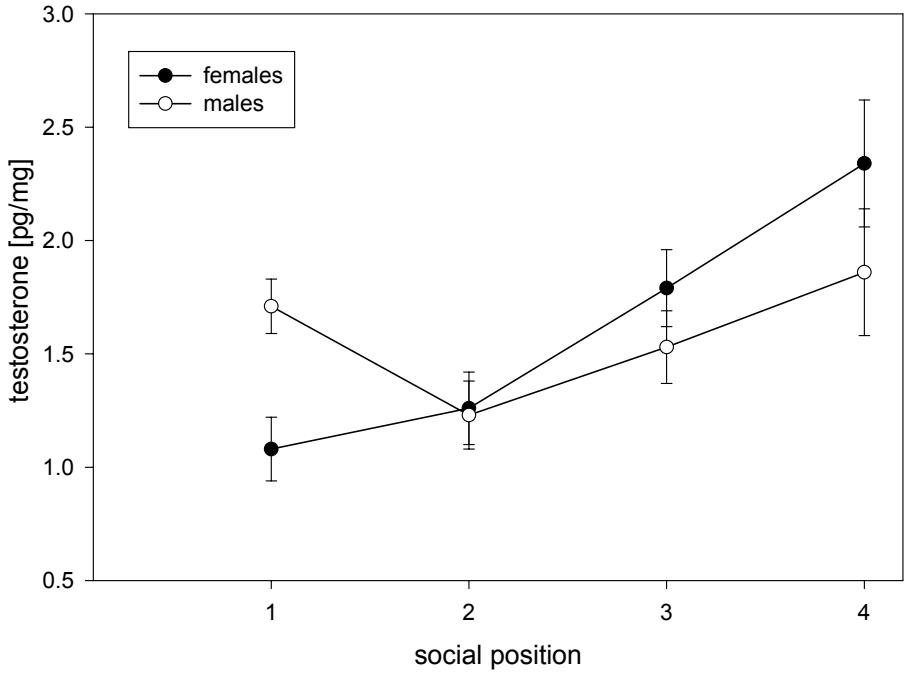

Figure 2: Testosterone concentrations in male (N=44) and female (N=46) eggs in relation to the social dominance status of the mother (1 = dominant, 2 = 1. intermediate, 3 = 2. intermediate, 4 = subordinate) (mean +/-std. error).

Discussion

(a) Sex determination and yolk hormones

This study showed that in White Leghorn chickens yolk hormone concentrations of testosterone and androstenedione did not differ significantly between male and female embryos after three days of incubation (figure 1). These results are in contrast to the data of Petrie *et al.* (2001), showing sex-specific differences in yolk androgen levels of peafowl eggs. There may be various reasons for the discrepancies between both studies. First, the different results between our study and that of Petrie *et al.* (2001) may be caused by species specific differences in maternal hormone allocation, which is unlikely since peafowl and chickens are closed related species. Second, if the sex-specific results in the peafowl are a consequence of secondary sex specific processes during early development, a difference in incubation time (10 days in the peafowl, 3 days in this study) might

explain the different results in these two studies (Elf & Fivizzanni 2000, but see also Eising *et al.* in prep). Third, the social position of the female, which we now demonstrate to be of significant importance, may have confounded the peafowl data.

When we included the social rank of the mother in our analysis we found a clear pattern for testosterone. With a decreasing position in the social hierarchy testosterone concentrations increased in female eggs but did not change in male eggs. Moreover, in eggs of dominant mothers testosterone concentrations were significantly higher for male eggs than for female eggs, while this was not the case for subdominant mothers. From a mechanistic point of view this raises the question how females can tell the sex of the offspring when providing the yolk with hormones, which would be necessary because sex of the embryo is determined after the yolk is formed (Sturkie 1986). Our results indicate that the androgens may be involved in sex determination, but that a simple causal link between yolk androgens and resulting offspring sex (Petrie et al. 2001) is unlikely (fig. 1). Our results indicate that if androgens (or a factor correlated with androgen levels) influence sex of the eggs they only do so in interaction with a factor that is linked to maternal social rank (fig. 2). This might be body weight (table 1), or other steroids. The latter suggestion fits with the data of two other studies. Petrie *et al* (2001) found that testosterone and androstenedione are higher while dihydro-testosterone and estradiol are lower in male than in female eggs. Bowden *et al.* (2000) found a similar result for turtles, although not at the level of the individual egg but at that of the whole clutch. Also stress hormones might be a relevant factor in this context since they have been shown to vary with social dominance and additionally to interact with reproduction (review in Creel 2001).

(b) Functional aspects of maternal hormone allocation

The sex-specific testosterone allocation fits the expectations of the sex allocation theory (see introduction). The family *Phasanidae*, which includes the ancestor of the domesticated chickens as well as peafowl, typically shows a polygynous mating system and a sexual size dimorphism with males larger than females (Glutz von Blotzheim 1973). In these polygynous mating systems male mating success is more variable than female mating success (e.g. Guhl & Warren 1946, Graves *et al.* 1985). In addition the larger sex is more expensive to rear due to higher food requirements (review in Anderson *et al.* 1993). Therefore, for parents with high rearing capacities increased investment in sons will have a greater impact on parental reproductive success than increased investment in daughters (Trivers & Willard 1973). Thus, parents capable of high levels of investment should either shift the sex ratio of their brood towards males or otherwise intensify the resource allocation to males. We did not find a shifted sex ratio in relation to the social dominance, which is in line with an earlier study on

chickens (Leonard & Weatherhead 1996). However, we found enhanced testosterone levels in male eggs relative to female eggs of dominant mothers. Since yolk androgens have been shown to increase competitiveness and growth (Schwabl 1996b, Eising *et al.* 2001), they provide a mechanism for adaptive maternal investment. Thus dominant females allocate particularly more to male offspring. Thereby they create a competitive asynchrony within their brood with an advantage for male chicks. This maternal favoritism probably enhances nutritional condition for sons, which has been shown to be of importance for males in the context of sexual selection (Gustafsson *et al.* 1995, De Kogel & Prijs 1996, David *et al.* 2000, Ohlsson *et al.* 2001). Especially nutrition early in the nestling phase, where maternal androgens are most likely to act, has a significant impact on the expression of sexual ornaments at adulthood and therefore reproductive success in a related species (ring-necked pheasant, Grahn & von Schantz 1994, Ohlsson *et al.* 2001).

Low-ranking females are probably restricted in the quantity of resources they can allocate to their offspring. In our experiment, the social status of a hen was negatively correlated with her body weight and mean egg weight in line with earlier findings (Collias 1943, Leonard & Weatherhead 1996), suggesting a reduction in her maternal capacities. Low-ranking females apparently are restricted in the amount of resources they obtain and thus can allocate to the eggs. Following the findings that maternal androgens have a beneficial effect on offspring growth (Schwabl 1993, Eising *et al.* 2001), subordinate females might try to compensate lower egg quality with an increasing amount of androgens in the yolk, balancing benefits against potential costs of testosterone (e.g. Sockman & Schwabl 2000). In line with sex allocation theory, they allocate more yolk androgens to the offspring with the lower variance in reproductive success, favoring daughters over sons.

In conclusion, this study clearly shows that it is very unlikely that androgen concentrations alone are the decisive part of the sex-determining mechanism. However, differential androgen deposition does take place in relation to both sex of the egg and social rank of the mother, and this might provide a mechanism for adaptive maternal investment.

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